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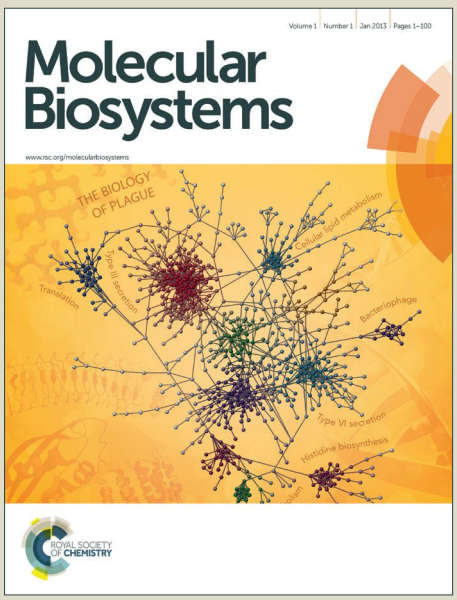

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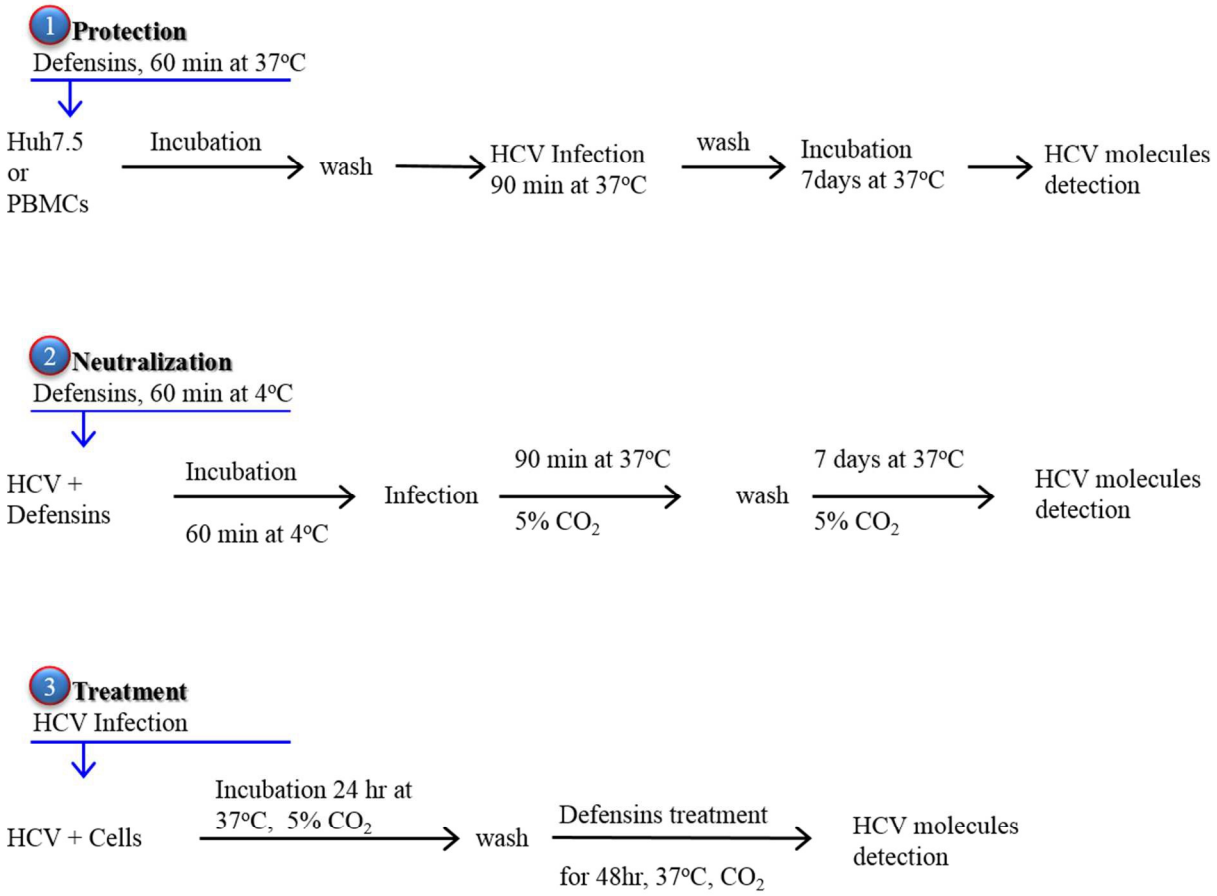

We revealed that human α- and β-defensins have strong anti-HCV activity in experiments on cellular protection, neutralization, and treatment at low concentrations, whereas synthetic linear avian defensins could reach similar anti-HCV potentials only at noticeably higher concentrations.

Virucidal activity of human α- and β-defensins against hepatitis C virus genotype 4

Ehab H. Mattar,[1] Hussein A. Almehdar,[1] Vladimir N. Uversky,[1,2,3,*] and Elrashdy M. Redwan[1,4,*]

[1] *Department of Biological Sciences, Faculty of Sciences, King Abdulaziz University, P.O. Box 80203, Jeddah, Saudi Arabia*

[3] *Department of Molecular Medicine and USF Health Byrd Alzheimer's Research Institute, Morsani College of Medicine, University of South Florida, Tampa, FL, USA;*

[3] *Laboratory of Structural Dynamics, Stability and Folding of Proteins, Institute of Cytology, Russian Academy of Sciences, St. Petersburg, Russian Federation;*

[4] *Therapeutic and Protective Proteins Laboratory, Protein Research Department, Genetic Engineering and Biotechnology Research Institute, City for Scientific Research and Technology Applications, New Borg EL-Arab 21934, Alexandria, Egypt.*

* Corresponding authors: Redwan, E.M. (redwan1961@yahoo.com), Uversky, V.N. (vuversky@health.usf.edu).

Abstract

Hepatitis C virus (HCV) is the major etiological agent of human non-A and non-B hepatitis affecting about 180 million people worldwide. The goal of current study was to find the effective anti-HCV proteins. As a result, defensins were selected as promising candidates due to their well-known anti-viral potentials and small size. We conducted *in vitro* evaluation of two kinds of defensins (human α- and β–defensins and synthetic linear avian α-defensins) using tissue culture combined with reverse transcription nested PCR (RT-nested-PCR) and real-time PCR. Human α- and β-defensins showed strong anti-HCV activity in experiments on cellular protection, neutralization, and treatment at all concentrations used (10, 20 and 50 μg). The synthetic linear defensins could reach similar anti-HCV potentials only at noticeably higher concentrations (250 μg) and do not show noticeable activity at 10 and 20 μg. This study suggest that defensins are potent anti-HCV agents.

Keywords: Hepatitis C virus; α-defensins; β-defensins; virucidal; linear defensins; intrinsic disorder; thermodynamic instability.

Introduction

The alternative and complementary medicine contain a number of means for HCV control. Among these means are defensins, short, cationic, cysteine-rich polypeptides that have pronounced biocidal activity and belong to a diverse group of antimicrobial peptides found in vertebrates, invertebrates, insects, and plants.[1-8] These polypeptides play important roles in innate immunity against microbial and viral infections, are involved in adaptive immunity, and play various roles in inflammation, wound repair, expression of cytokines and chemokines, production of histamine, and enhancement of antibody responses.[9-11] They are also able to induce and augment antitumor immunity when fused with the non-immunogenic tumor antigens.[12] These 28–42 amino acid cationic peptides are assumed to possess a conserved fold and contain six highly conserved cysteine residues, which form three pairs of intramolecular disulfide

bonds, specific patterns of which are well-preserved during the evolution.[8, 13-15] Based on their cellular origin, the spacing between the cysteine residues, and the number and pattern (or topology) of their disulfide bridges, the vertebrate defensins are classified as α-, β-, and γ-defensins.[8, 15, 16] In mammals, barrier epithelial cells mostly generates β-defensins, whereas α-defensins are mainly stored in the azurophil granules of neutrophils.[7] In the mouse, Paneth cells and skin produce at least 17 α-defensins, whereas various epithelial cells and keratinocytes generate four β-defensins.[17]

In this study, several innate immune defense peptides and proteins of different nature were analyzed for their potential activities against hepatitis C virus (HCV) using the *in vitro* culture system. We analyzed: proteins purified from natural resources (human neutrophils peptides, α-defensins 1 to 4 as mixture); recombinant proteins (human β-defensins 1 to 5 and 116 as mixture), and synthetic linear peptides (avian β-defensins AvBD-4, AvBD-7, AvBD-12). Their antiviral activities were monitored in peripheral blood mononuclear cells (PBMCs) and Huh7.5 cell line using three experimental strategies depicted in Figure 1 that are based on two main methodologies for detection of the viral molecules, reverse transcription nested PCR (RT-nested-PCR) and real-time-PCR. Throughout the study, all experiments has been run in duplicate, unless otherwise mentioned. We also evaluated the total concentrations of α- and β–defensins in HCV-infected patients and non-infected subjects using commercial ELISA kits.

Figure 1.

RESULTS

Cell viability and cytotoxic effects of defensins

First, cytotoxic effects of different defensins on PBMCs and Huh7.5 cell line were studied. To this end, the PBMCs (2.5×10^5) and Huh7.5 cells (10^5) treated for 7 days with defensins at the maximal concentrations to be used in the antiviral activity screening (50 and 250 µg/ml) were compared with the untreated PBMCs and Huh7.5 cultures. This analysis revealed that human α–defensins were not cytotoxic, whereas

at their highest concentrations, human β–defensins and avian synthetic defensins caused a slight reduction in the viability of both cells, to ~93-95% (Table 1).

Table 1.

Evaluation of the anti-HCV activity of defensins using RT-nested PCR

Cell protection by defensins against the entry of HCV particles

As shown in Figure 1, both Huh7.5 and PBMC cell cultures were treated with human α- or β-defensins or avian defensins for 60 min, then washed three times with the PBS buffer or fresh medium, and then infected with HCV for 90 min. The inoculated cells were cultured for seven days. At all concentrations tested, α-defensins were effective protectors of both cell types against the HCV attack. β-Defensins also efficiently protected cells at concentrations of 20 and 50 µg/ml but completely failed to do so at 10 µg/ml. Synthetic linear avian β-defensins (AvBD-4, 7, and 12) failed to protect both cell cultures from the HCV entry at lower concentration 10 and 20 µg/ml and showed protection only at very high concentrations of 250 µg/ml (Figure 2A). Figure 2A shows that the HCV-related band of 174 bp was not amplified in all protected cells and amplified in non-protected cells. Finally, camel lactoferrin (cLac) was used as a positive control, whereas Rulc system was used as quality and reproducibility indicator of the amplification system.

Figure 2.

Defensins neutralization potentials against HCV particles

Next, defensins were tested for their HCV neutralization potentials. To this end, they were incubated with HCV-infected serum at concentrations of 10, 20, 50 and/or 250 µg/ml for 60 min, and these pre-incubated mixtures were used to infect Huh7.5 or PBMCs cells. After incubation for 90 min the cell cultures were washed three times

with PBS or fresh media and the inoculated cells were cultured for seven days. Figure 2B shows that human α- and β-defensins were able to completely neutralize all HCV particles and subsequently inhibit the viral entry into the cells at all concentrations. On the other hand, the avian β-defensins failed to neutralize and block the HCV entry into cells at concentrations of 10 and 20 μg/ml and were able to do so only at the highest concentration of 250 μg/ml.

Effect of the intracellular treatment with defensins on HCV replication

Human defensins at concentrations of 10, 20, and 50 μg/ml and avian defensins at concentrations of 10, 20, 250 μg/ml were investigated for their *in vitro* ability to inhibit the viral replication inside the infected Huh7.5 and PBMCs cells. Inhibition of viral replication was detected by amplification of viral non-coding RNA segments using the RT-PCR technique. Human defensins at all concentration tested were able to completely inhibit the HCV replication in the Huh7.5 cells and in the PBMCs (Figure 2C) within 48 h. However, avian β-defensins did not inhibit HCV replication at any concentrations used (10, 20, and 250 μg/ml) (Figure 2C).

Evaluation of the anti-HCV activity of defensins using real time PCR

In addition to the RT-nested-PCR used for detection of HCV presence in the cells, we also utilized the real-time-PCR to detect and measure the HCV copy number throughout all conditions analyzed in this study.

Cells protection by defensins against entry of HCV particles

The HCV copy number calculations revealed that at concentrations of 10, 20 and 50 μg/ml, human α-defensins were able to protect the PBMCs and Huh7.5 cells from attack by the HCV viral particles since no HCV particles were found in these experiments, indicating a relative activity of 100% for these defensins. Human β-defensins also offered a comparable protection for both cells types but possessed the relative activity of 100% only at concentrations of 20 and 50 μg/ml, whereas at 10

μg/ml these peptides were somewhat less potent protecting both cell types against HCV entry (see Table 2). The avian defensins reached the relative activity of 100% only at the concentrations of 250 μg/ml in both PBMCs and Huh7.5 cells, whereas the protective effects of lower concentrations of these defensins were noticeably less pronounced.

Table 2.

Neutralization potentials of defensins against HCV particles

Based on the HCV copy number calculations, it was clear that natural human α-defensins and human recombinant β-defensins were able to totally neutralize the HCV particles and subsequently protect the PBMCs and Huh7.5 cells from the HCV infection at all concentration studied (see Table 3). On the other hand, avian synthetic β-defensins neutralized all HCV particles only being added at concentration of 250 μg/ml, whereas at lower concentrations they showed low neutralization activity and their neutralization activity was concentration dependent.

Table 3.

Effects of intracellular treatment with defensins on HCV replication

Table 4 shows that according to the HCV copy number calculations, human defensins were able to penetrate to the pre-infected Huh7.5 cells and PBMCs and completely blocked the HCV genome replication and the subsequent assembly of viral particle at all concentrations studied. However, synthetic avian β-defensins were much less efficient in penetration of the infected Huh7.5 cells and PBMCs, and, consequently, significant numbers of viral particles were seen at all concentrations of these defensins studied in this work. The highest relative activity was achieved by 250 μg/ml of AvBD-12 (21% and 30.6% in infected Huh7.5 cells and PBMCs). Noticeably, although low, the ability of avian β-defensins to affect HCV replication

was typically concentration dependent and increased with the increase in the content of corresponding defensin.

Table 4.

DISCUSSION

Hepatitis C virus is an enveloped, single-stranded positive-sense RNA virus that belongs to the *Flaviviridae* family. There is no insect vector or animal reservoir for HCV, and the virus is acquired through person-to-person transmission by parenteral routes (i.e., in a manner other than through the digestive tract).[18] Before clinical screening for HCV became available, infection was mainly transmitted by transfusion of contaminated blood or blood products. Nowadays transmission frequently occurs through the use of contaminated needles, syringes, and other instruments used for injections and other skin-piercing procedures. Sexual transmission of hepatitis C occurs rarely.[19]

HCV is the major cause of parenterally transmitted non-A and non-B hepatitis worldwide,[18] and infection with HCV is one of the leading causes of chronic liver disease worldwide.[20] The prevalence of HCV infection has increased during recent years. It is estimated now that over 180 million people are infected with HCV world-wide. This means that 3% of the world's population are affected by HCV, and in some countries, such as Egypt, this number reaches 15%.[21] More than 70% of patients infected with HCV develop chronic, if not lifelong, infection. Furthermore, persistent HCV infection accounts for ~50% of serious end-stage liver diseases, such as liver cirrhosis, hepatic failure, and hepatocellular carcinoma.

There are six major genotypes of HCV found throughout the world, with genotypes 1, 2, 3 and 4 being further subdivided on several sub-genotypes. Many HCV genotypes are unevenly distributed, with genotypes 1 and 3 being found in most countries irrespective of their economic status, and with the largest number of incidences of genotypes 4 and 5 being reported in lower-income countries.[22] Major clinical research on antiviral therapy for chronic HCV has been conducted in Western countries[23] and

in Japan.[24] Therefore, most published data deal with patients infected with HCV genotypes 1, 2, and 3, and there are now articulate guidelines for the type of treatment and period of antiviral treatment in such patients.[25] However, there have been relatively few studies that deal with the patients infected with HCV genotype 4 (which is highly prevalent in North Africa and the Middle East), and combination therapy trials (interferon and ribavirin) for these patients did not demonstrate promising efficacy.[26]

Currently, no vaccine is available to prevent HCV infection. Standard treatment with interferon and ribavirin remained a gold standard of the chronic HCV remedy. This therapy achieves 50% sustained virological response (SVR, which is aviremia 24 weeks after completion of antiviral therapy) for genotype 1 and 80% for genotype 2 and 3. Recent studies have shown that HCV genotype-4 patients have a response rate to pegylated interferon monotherapy or combination interferon-ribavirin therapy that is less favorable compared to genotypes 2 and 3, and the response failure rate of about 50% is observed. As pegylated interferon is expensive, standard interferon is still the main therapy for HCV treatment in under-developed countries.[27] Furthermore, It is recognized now that the combined pegylated interferon-ribavirin therapy might have severe side effects, such as haematological complications.

In addition to interferon and ribavirin, there are several FDA-approved anti-HCV drugs. The emerging novel antivirals should optimize the treatment options, especially for difficult-to-treat patients, such as those who are suffering from advanced liver diseases or other co-infections, and who have poor response rates to current regimens. Although the currently approved and used cocktail of anti-HCV therapy is believed to cure more than 90% of infected patients, the appearance of viral resistance (due to the error-prone replication of this RNA virus), the presence of non-responders or treatment failure, superimposed with the adverse effects caused by the drugs in addition to treatment cost, are still major limitations that must be resolved. Furthermore, most entry inhibitors target host components, such as receptors or key enzymes, which are required for HCV entry and definitely have high genetic barriers to resistance due to their conserved nature. Therefore, these inhibitors tend to not only have pan-genotypic activity against virus infection but also possess a greater risk of simultaneously causing cellular toxicity.[28]

This work was dedicated for the *in vitro* evaluation of the antiviral potentials of human α- and β-defensins against HCV. For evaluation of the efficiency of anti-HCV candidate agents, three strategies were followed, cellular protection, viral particle neutralization, and intracellular viral replication inhibition in two *in vitro* models, peripheral blood mononuclear cells (PBMCs) and Huh7.5 cell line, using RT-nested-PCR and real-time PCR as the most accurate methodologies suitable for these analyses. This analysis revealed that natural human α-defensins (HNP-1, HNP-2, HNP-3, and HNP-4) and recombinant human β-defensins (1 through 5 and 116 as mixture) have a relative anti-viral activity of 100% in all three experimental settings (cellular protection, viral particle neutralization, and intracellular viral replication inhibition) at all concentrations studied (10, 20 and 50 μg/ml). The only exception from this general trend was the case of the lowest concentrations of human β-defensins (10 μg/ml) that could not completely protect the Huh7.5 cells, possessing a relative activity of 59%. Generally, human α- and β-defensins were able to completely neutralize all HCV particles added and subsequently inhibit the viral entry into the Huh7.5 and PBMC cells. Furthermore, there were no markers indicating the presence of the HCV amplified band or HCV particles within these cells. Different situation was observed for the synthetic linear avian β-defensins (AvBD-4, AvBD-7, and AvBD-12) that failed to protect both cell cultures from the HCV entry and were unable to neutralize viral particle and inhibit intracellular viral replication at concentration 10 and 20 μg/ml, being able to show antiviral activities at much higher concentrations (250 μg/ml). It is important to note that concentrations of human α- and β-defensins used in this study (10 – 50 μg/ml) were within the biologically relevant range. In fact, it is known that the levels α-defensins in the human plasma range from 400 ng/ml in healthy individuals to 13 μg/ml in individuals with bacterial infections, and may be as high as 6 mg/ml within neutrophils.[29, 30]

Unfortunately, we could not find any systematic study on the antiviral activities of human defensins against HCV. The only exception is the poster of Sherker *et al*. presented at The International Liver Congress 2012 – 47th Annual Meeting of the European Association for the Study of the Liver.[31] These authors analyzed cellular protection and inhibition of the intracellular viral replication using only α-defensin 1 (HNP-1), whereas we looked at the cellular protection, viral particle neutralization, and inhibition of the intracellular viral replication delivered by the purified mixture of

native human α-defensins HNP-1, HNP-2, HNP-3, and HNP-4 and recombinant human β-defensins (1 through 5 and 116 used as a mixture). Furthermore, the authors of the previous study used the HCV cell culture (HCVcc) system in Huh7.5.1 cells and HCV pseudo-particle (HCVpp) and assessed viral translation and replication with specific HCV replicons.[31] Whereas we used natural infection replication system with native HCV genotype-4a, since this better reproduces the biology and kinetics of HCV infection, where the HCV particles infect the hepatocytes and produces infectious viral particles. Furthermore, the patient serum contains the whole viral particle with all its quasi-species of different infectivity magnitudes whereas the fabricated HCV RNA particles (HCV pseudo-particles) are usually homogenous. However, despite the numerous methodological differences, the results of both studies are rather similar and mutually supportive.

Since Sherker *et al*. did not analyze blocking/neutralization efficiency of defensins against HCV[31] and since we could not find any published work on the effects of β-defensins against not only HCV but any other member of the *Flaviviradae* family and against viruses close to HCV within the genus *Pestivirus*, our work is the first study where the direct interactions between human α- and β-defensins and HCV viral particles were analyzed. In fact, we have established that the activity of α- and β-defensins against HCV was mediated primarily by the effect of these peptides on both the target cell and the viral particles. Our results are consistent with previous reports on the presence of such activity of defensins against other types of viruses. For example, the ability of HNP-1–3 to directly inactivate HSV and other enveloped viruses, including influenza A virus was reported, suggesting the ability of defensin to destabilize viral envelopes.[32] Also, the activity of HNP-1-3 defensins against HIV was reported,[33] and defensins were shown to inhibit infectivity of the number of enveloped viruses, such as vesicular stomatitis virus (VSV), cytomegalovirus, influenza A virus (IAV), sindbis virus, vaccinia virus, baculovirus, and herpes simplex virus (HSV), as well as some non-enveloped viruses including human adenovirus (HAdV), adenoassociated virus (AAV), and human papillomavirus (HPV).[34]

It remains unclear exactly how defensins alter host cells. *In vivo*, an antiviral role of defensins may be manifested by affecting innate and adaptive immune responses. Some defensins block viruses by up-regulating type I interferon response genes, whereas β-defensins may also act as chemoattractants for T-cells, monocytes, mast

cells, and dendritic cells. Defensins can also activate intracellular signaling networks to induce immune cell maturation, cytokine secretion, and antibody production.[35] The nanomolar concentrations of α-defensins are chemotactic for human monocytes and immature dendritic cells.[36] α-Defensins induce interleukin (IL)-8 release *in vitro*[37] and enhance the synthesis and secretion of IL-8[37-39] and IL-1 in airway epithelial cells and primary bronchial cells.[38] Other reports have shown that α-defensins are able to enter the cells[40] possibly by binding to the low-density lipoprotein receptor–related protein/α2-macroglobulin receptor and inhibits PKCα/β by direct binding to this kinase.[41] In agreement with these observations, HNP-1 is among the most potent inhibitory peptides of PKC.[42] Therefore, one of the possible mechanisms by which defensins inhibits replication of viruses involves interference with the PKC-mediated inhibition of viral entry. This is in line with our finding showing that the inhibition is observed when human defensins were added soon after infection. Finally, although the direct membrane disruption by defensins is considered as one of the potential molecular mechanisms of their anti-bacterial, anti-fungal, and anti-parasitic action[43] such mechanism cannot be easily applied to enveloped and non-enveloped viruses.

Therefore, several mechanisms of action of defensins against enveloped and non-enveloped viruses can be proposed:

1. Direct distortion of the viral envelope through perturbation of the viral lipid membranes. This model is not consistent with the previous reports.[44]
2. Charge-charge attraction of defensins to viruses. It is unlikely that this mechanism is dominant in the antiviral activity of defensins, since this antiviral activity is generally preserved at physiological salt concentrations, whereas the linearized α-defensins that lack a disulfide-stabilized 3-D structure are nonfunctional against all viruses tested.[34] Furthermore, although β-defensins are, on average, more charged than α-defensins, they typically exhibit less antiviral activity, especially against non-enveloped viruses.[34, 45]
3. Immunomodulatory role,[35] where defensins participate in activation and/or enhancement of the functions of immune cells recruited to a site of viral infection.[45]
4. The ability to distort/modulate structures of viral proteins. Recent study indicated that the intrinsic disorder as well as thermodynamic instability of microbial proteins are the decisive characteristics of protein susceptibility to interaction with defensins.[46] Since viral proteomes in general contain numerous intrinsically disordered proteins,[47-

[50] and since many HCV proteins are intrinsically disordered,[51] it is likely that this intrinsically disordered nature of HCV proteome make its proteins susceptible for defensins.

Curiously, Figure 3 and Table 5 show that defensins themselves contain significant amounts of intrinsic disorder, with human proteins being, in general, noticeably more disordered than avian polypeptides. The disorderedness of these proteins is evident from their high mean disorder scores (see Table 5), and from the presence of disordered tails (Figure 3). Furthermore, Figure 3 shows that defensins analyzed in this study can be grouped into three sets based on the peculiarities of their per-residue disorder profiles obtained by PONDR® FIT, which is a metapredictor of intrinsic disorder that is known to be moderately more accurate than each of the component predictors.[52] This disorder-based grouping of defensins coincides with the traditional classification of these proteins, suggesting that different classes of defensins are characterized by class-specific peculiarities of disorder distributions. We also looked at the disorder propensity of defensins by classifying them as mostly ordered or disordered proteins using charge-hydropathy plot (CH-plot).[53, 54] This approach is able to discriminate proteins with substantial amounts of extended disorder (random coils and pre-molten globules, which are located above the boundary in the corresponding CH-plot) from proteins with globular conformations (molten globule-like and ordered globular proteins, which are positioned below the boundary).[53] Figure 3D shows that human β-defensins are noticeably more disordered than human α-defensins and avian β-defensins. Points corresponding to human β-defensins-3, -4 and -116 are located above the boundary separating compact proteins and extended disordered proteins, indicating that these three defensins are predicted to have extended disordered structures. Although points corresponding to human β-defensins-1, -2, and -5 are located below this boundary, they are noticeably closer to the boundary than points corresponding to human α-defensins and avian β-defensins that have comparable charge-hydropathy attributes (Figure 3D).

Figure 3

Table 5

In summary, we report here unique data on the ability of human native and recombinant defensins to protect cellular systems from the HCV attack, to neutralize viral particles, and to inhibit intracellular viral replication. These important observations, taken together with the fact that the serum of HCV patients contains highly elevated levels of defensins, clearly indicates that the pharmaceutical potentials of human defensins cannot be ignored, especially considering their strong antiviral activity combined with low molecular weight, reduced immunogenicity and antigenecity, broad biocidal spectrum, and resistance to proteolysis.

MATERIALS & METHODS

Samples

Samples of the HCV-infected human serum and/or plasma used in our research (without patient name(s) or medical history) were supplied by the ALBOURG clinic lab (Giza, Cairo Egypt) under supervision of Prof. Ehab Eldab. Samples from ten hepatitis C patients with high viremia (8.3 million copies/ml) positive for viral genotype 4 antibody and confirmed by PCR, were used for the *in vitro* infection experiments. The peripheral blood leucocytes (PBLCs) used in our study were from these volunteers, to whom the goals of the experiments were explained and whose informed consent was obtained in a form of oral approval. All experiments were performed in compliance with the relevant laws and institutional guidelines.

For *in vitro* infection experiments, we utilized serum samples positive for the HCV antibody and RNA as determined by ELISA and RT-nested PCR and genotyped as genotype-4 using the method described in ref.[55]

Human subjects provided informed consent

Chemicals

Chemicals were purchased from Fluka Biochemika (Buchs, Switzerland), Amersham Pharmacia Biotech (Uppsala, Sweden), Riedel-deHaen (Germany), WINLAB (U.K.), Sigma chemicals Co. (St.Louis, Mo., USA), Acros (New Jersey, USA), PARK (Northampton, U.K.), Fischer Scientific (U.K.), Scharlau Chemie S.A. (Barcelona, Spain), and Athen Research and Technology (Virginia, USA).

Agarose was from GEBCO BRL (Paisley, Scotland), RPMI-1640 cell culture media were purchased from HyClone (Logan, Utah), fetal bovine serum, penicillin, streptomycin, and trypsin were obtained from Sigma. Primers for PCR and $MgCl_2$ were purchased from Clontech (USA); dNTP and Taq DNA polymerase were purchased from Promega (Madison, WI, USA); Ready-To-Go RT-PCR beads was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA), DNA ladder obtained from Promega (USA), Thiazolyl Blue Tetrazolium Bromide was purchased from Sigma-Aldrich Chemie GmbH (Germany). The Huh7.5 cell line was as a gift from Professor Carl Rice (USA).

Defensin proteins and peptides

Natural human α–defensins known as human neutrophil peptides HNP-1, HNP-2, HNP-3, and HNP-4 were purified from human neutrophils. Recombinant human β-defensins (1-5 and 116) were produced in prokaryotic expression system. Avian β-defensins (AvBD-1, AvBD-2, and AvBD-3) were obtained as linear peptides via the *in vitro* synthesis. Sequences and basic physico-chemical properties of defensins used in this study are listed in Table 5.

Cell cultures of PBMCs

Peripheral mononuclear blood cells (PBMCs) were isolated as reported in ref.[56] Briefly, peripheral blood samples from healthy individuals were diluted with 5 volumes of a freshly prepared RBC lysis buffer (38.8 mM/L NH_4Cl, 2.5 mM/L K_2HCO_3, 1 mM/L EDTA, pH 8.0), incubated at room temperature for 10 min and centrifuged at 1,500 rpm and 4ºC for 5 min. The nucleated cells were precipitated in the bottom of the tube. The pellet was collected and washed three times with PBS.

425

426 **Assays for the analysis of cytotoxic effects of defensins**

427 Throughout the current study, all *in vitro* experiments with the cultured tissue cells
428 were run in duplicates. The cytotoxic effect of defensins on PBMCs and Huh7.5 cells
429 was examined by the counting of viable cells after trypan blue treatment and by the 3-
430 (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The
431 Huh7.5 cells were washed twice in RPMI-1640 media supplemented with 200 µM L-
432 glutamine in 25 µL HEPES buffer (N-[2-hydroxyethyl] piperazine-N-[2-
433 ethanesulphonic acid]). PBMCs cells (2.5×10^5) and Huh7.5 (1.0×10^5) were plated in
434 three 24-well microtiter plates in duplicates and cultured in RMPI-1640 culture media
435 (RMPI-1640 supplemented media, 10% fetal bovine serum (FBS), 100 U of penicillin
436 and 100 µg streptomycin) for two days at 37ºC, 5% CO_2 and 88% humidity before
437 defensins treatment, then the medium was refreshed with new RMPI-1640
438 supplemented medium containing 100 µg/ml of defensins. The cells and defensins
439 were incubated for 90 min at 37ºC and washed three times with 1 ml of PBS. The
440 cells were maintained in 1 mL of fresh medium for seven days at 37ºC, 5% CO_2 and
441 88% humidity. After one week of culture, the cells were collected and suspended in
442 medium. For collection of Huh7.5 cells, the adherent cells were detached from the
443 plate using 200 µL trypsin/EDTA mixture (200 mg/L EDTA, 500 mg/L trypsin in a
444 ratio 1:250) for 1–3 minutes, the action of trypsin was stopped by the addition of 1
445 mL of RPMI culture media. The cells were scrapped and collected in a 15 ml falcon
446 tube, then washed twice by RPMI supplemented media and once by phosphate buffer
447 saline (PBS), and centrifuged at 1,000 rpm for 5 min after each wash. The pellet was
448 resuspended in 1.0 ml PBS, and then the total number of viable cells was counted
449 using the trypan blue treatment. We also examined the viability of cells which were
450 cultured for one day with medium containing 50 and 100 µg/mL of defensins.

451 The viability of the cells was also assayed by Thiazolyl Blue Tetrazolium Bromide
452 (MTT) method as following: 10×10^3 PBMC cells or Huh7.5 cells in 200 µL culture
453 media per well were placed in a 96-well plate. Plate was incubated at 37ºC, 5% CO_2
454 and 88% humidity before defensins treatment, then the medium was refreshed with
455 new RMPI-1640 supplemented medium containing 0.100 mg/mL of defensins. The
456 cells and defensins were incubated for 90 min at 37ºC and washed three times with

200 µl of PBS. The cells were maintained with 200 µL of fresh medium for seven days at 37ºC, 5% CO_2 and 88% humidity. Then, 20 µl MTT solution (5 mg of Thiazolyl Blue Tetrazolium Bromide (MTT) per 1 ml PBS buffer) was added to each well. Plate was placed on a shaking table, shaken at 150 rpm for 5 min, to thoroughly mix the MTT into the media. Then, the plate was incubated at 37ºC, 5% CO_2 and 88% humidity for 5 hours to allow the MTT to metabolize. Next, aliquots of 200 µL of dimethylsulfoxide (DMSO) were added to each well, and plate was placed on the shaking table, shaken at 150 rpm for 5 min and then used to read optical density at 595 nm by ELISA reader. The viability of cells which were cultured for one day with medium containing 50 and 100 µg/ml of defensins was also examined.

Antiviral activity of natural human α-defensins (HNPs), recombinant human β-defensins (RHBDs), and synthetic linear avian β-defensins (ABDs) against HCV

The antiviral activities of human natural α-defensins (human neutrophil peptides, α-defensins 1 to 4 as a mixture), human recombinant β-defensins (RHBD-1, RHBD-2, RHBD-3, RHBD-4, RHBD-5, and RHBD-116 as a mixture), and synthetic linear avian β-defensins (AvBD-4, AvBD-7, AvBD-12) were analyzed. Their antiviral activities were monitored in peripheral blood mononuclear cells (PBMCs) and Huh7.5 cell line using three experimental strategies, such as cellular protection, viral particle neutralization, and inhibition of intracellular viral replication depicted in Figure 1, and two methodologies were used for detection of the viral molecules, RT-nested-PCR and real-time-PCR.

There is a clear difference in the action of defensins in different experiments used in this study, despite the fact that there could be an overlap between the mechanisms of defensin action in these three types of experimental approaches. In the "Cell protection by defensins against the entry of HCV particles" approach, cells are first treated with defensins and then there pre-treated cells are exposed to the virus. Therefore, it is expected here that defensins act directly on cells. In the "Defensins neutralization potentials against HCV particles" approach, the infected serum is first treated with defensins and then this infected serum pre-treated with defensins is used to infect cells. Therefore, in this approach, defensins are expected to act directly on HCV particles. In the "Inhibition of intracellular viral replication" approach, HCV-

infected cells are treated with defensins. Although one cannot exclude scenario, where defensins can act on the HCV particles inside infected cells, there is also a possibility that in this approach defensins possess effects on some cellular mechanisms and pathways, potentially acting as cytokines or growth hormones. Exact mechanisms of the intracellular activity of defensins are not know and this topic requires further work.

Protection potential of natural human α-defensins (HNPs), recombinant human β-defensins (RHBDs), and synthetic linear avian β-defensins (ABDs) on HCV

To examine the cellular protection effects of human α-defensins (HNPs), recombinant β-defensins (RHBD), and Avian β-defensins (ABD1-3), multiple parallel cultures the human PBMCs (2.5×10^5) and Huh7.5 (1.0×10^5) cells were plated in three 24-well microtiter plates. HNPs, RHBDs, or ABD1-3 were added to the PBMCs and Huh7.5 cells in 50 mL of RPMI-1640 supplemented medium at a final concentration of 10, 20, or 50 μg/ml for each the above defensin peptides, then incubated for 60 min at 37ºC. Free defensins were removed by washing the cells three times with 1 mL of cold PBS. After addition of 10 mL of medium containing 1 mL of HCV-infected serum (8.3 million copies/mL, RNA G4), the cells were incubated for 90 min at 37ºC. The cells were then washed three times with PBS and cultured for seven days at 37ºC, 5% CO_2 and 88% humidity,[57, 58] followed by total RNA extraction to use in RT-nested-PCR and real-time PCR.[59]

Neutralization potential of HNPs, RHBDs, and synthetic linear ABDs on HCV

To examine the neutralization effects of natural HNPs, RHBDs, or synthetic ABD1-3 on the HCV, one mL of infected serum (8.3 million copies/mL, RNA G4) and HNPs, RHBDs, or ABD1-3 at final concentration 10, 20, or 50 μg/ml, according to refs.[60, 61] was pre-incubated in 10 ml of medium for 1 h at 4ºC, and then the mixtures of HCV and defensin peptides were added to PBMCs and Huh7.5 cells cultured as described above, and incubated for 90 min at 37ºC, 5% CO_2 and 88% humidity. The cells were washed three times with 1 mL of PBS and further incubated for 7 days at 37ºC, 5% CO_2 and 88% humidity. Virus-positive cells (PBMCs (2.5×10^5) and Huh7.5 (1.0×10^5

infected with HCV) and virus-negative cells (PBMCs (2.5×10^5) and Huh7.5 (1.0×10^5) cells without infection) were included in the assay. The cells were washed three times from debris and dead cells using RPMI-1640 supplemented media, followed by total RNA extraction, [59] to use in the RT-nested-PCR and real-time PCR experiments.

Treatment potential of natural HNPs, RHBDs, and synthetic linear ABDs on HCV

Huh7.5 cells were washed twice in RPMI 1640 supplemented media. The cells 2×10^5 cells/mL in RPMI 1640 culture media (RPMI-1640 supplemented media, 10% fetal bovine serum (FBS); 100 U of penicillin and 100 µg streptomycin) were added to two sets of 12-well plates and left to adhere for 24 h at 37ºC, 5% CO_2 and 88% humidity. Then, cells were infected with the HCV-infected serum (8.3 million copies/mL, RNA G4) in RPMI-1640 media and incubated for 48 h at 37ºC, 5% CO_2 and 88% humidity. The defensins were added at concentrations of 10, 20, or 50 µg /ml. Positive Huh7.5 (2×10^5) cells infected with HCV and negative Huh7.5 (2.0×10^5) cells without infection were included in these experimenst. The cells were incubated for 4 days at 37ºC, 5% CO_2 and 88% humidity. Camel lactoferrin at concentration of 0.5 mg/ml was used as a positive inhibitor of HCV infection.[57] The cells were washed three times from debris and dead cells using RPMI-1640 supplemented media, followed by total RNA extraction,[59] to be used in the RT-nested-PCR and real-time PCR analyses.

Extraction of RNA from PBMCs and Huh7.5 cells

RNA was isolated from PBMCs and Huh7.5 cells as reported in ref.[56] Briefly, cells from different experiment were precipitated by centrifugation at 1,500 rpm for 5 min at 4ºC and washed thoroughly with PBS or basal media to remove adherent viral particles before lysis in 4 mol/L guanidinium isothiocyanate containing 25 mM sodium citrate, 0.5% sarcosyl, 100 mM β-mercaptoethanol, and 100 µL sodium acetate. The lysed cells were centrifuged on a microcentrifuge (Heraeus Sepatech, Germany) at 12,000 rpm for 10 min at 4ºC. The aqueous layer was collected and mixed with equal volume of isopropanol. After incubation at -20ºC overnight, RNA

was precipitated by centrifugation at 12,000 rpm for 30 min at 4ºC and the precipitated RNA was washed twice with 70% ethanol.

RNA for the internal controls was synthesized as described in ref.[59] Briefly, RNA encoding *Renilla* luciferase (Rluc) was used as an internal control to monitor the efficiency of RT-nested PCR. The pRL-TK plasmid vector encoding Rluc was linearized by cutting at the Xba I site and then used as a template for *in vitro* transcription with T7 RNA polymerase.[62] The synthesized RNA was treated with DNase and purified using an RNaeasy Mini kit.

Detection of HCV by RT-nested-PCR

Reverse transcription-nested PCR was carried out according to ref.,[56] with few modifications. The complimentary DNA (cDNA) and the first PCR reaction of the nested PCR detection system for the HCV and rluc RNA were performed in a 50 µL volume single-step reaction using the Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech, Pis-cataway, NJ, USA), 400 ng of total RNA, 10 µM of the reverse primer 1CH (for plus strand), 10 µM of the forward primer 2CH (for minus strand), and 10 µM of reverse primer P2. The test was incubated at 42ºC for 60 min and denatured at 98ºC for 10 min. Amplification of the highly conserved 5'-UTR sequences was done using two PCR rounds with two pairs of nested primers (Clontech, USA). First round amplification was done in 50 µL reaction mixture containing 10 µM of each of 2CH forward primer and P2 reverse primer, 0.2 mM/L of dNTP, 5 µL of RT reaction mixture as template and 2 U of Taq DNA polymerase (Promega, Madison, USA) in a 1×buffer supplied with the enzyme. The thermal cycling protocol was as follows: 1 min at 94ºC, 1 min at 55ºC, and 1 min at 72ºC for 30 cycles. The second round amplification was done similar to the first round, except for use of the nested reverse primer D2 and forward primer F2 at 10 µM each. A fragment of 174 bp was identified in positive samples. Primer sequences were as follows:

1CH: 5'-GGTGCACGGTCTACGAGACCTC-3';
2CH: 5'-AACTACTGTCTTCACGCAGAA-3';
P2: 5'-TGCTCATGGTGCACGGTCTA-3';

D2: 5'- ACTCGGCTAGCAGTCTCGCG-3';
F2: 5'-GTGCAG CCTCCAGGACCC-3'.

To avoid the reduction of the efficiency of HCV amplification reaction, cDNA was amplified with 5:1 HCV-to-Rluc primer concentrations in the first and second rounds of PCR. To control false detection of negative-strand HCV RNA[55, 56] and known variations in PCR efficiency, specific control assays and rigorous standardization of the reaction were employed. Specific control assays were included:

(1) cDNA synthesis without RNA templates to exclude product contamination;
(2) cDNA synthesis without RTase to exclude Taq polymerase and RTase activity;
(3) cDNA synthesis and PCR step done with only the reverse or forward primer to confirm no contamination from mixed primers.

These controls were consistently negative. In addition, cDNA synthesis was carried out using only one primer followed by heat inactivation of RTase activity at 95ºC for 1 h, in an attempt to diminish false detection of negative-strand prior to the addition of the second primer. Amplified DNA (174 bp for HCV and 376 bp for Rluc) were detected by staining with ethidium bromide after separation on a 3% agarose gel electrophoresis.[57, 63]

Quantification of HCV loads by real-time PCR

Briefly, HCV RNA was extracted from PBMCs and Huh7.5 cells as described above. Amplification of HCV RNA in samples and standards was measured by SYBR Green kit with two-step PCR, where the RNA is first reverse-transcribed into cDNA using 1CH, 2CH and P2 primers, then the second step takes place with D2 and F2 primers. An aliquot of the reverse transcription reaction is then used for analysis of viral load using the Rotor-Gene real time PCR machine and the report was generated by Rotor-Gene Q Series Software 1.7 (Build 94) Copyright© 2008 Corbett Life Science, a QIAGEN. As described previously in refs.[57, 58] the relative activity (%) was calculated as [(A) count of positive control – (B) count of tested protein]/(A) count of positive control × 100%.[57, 63]

Statistical analysis

Throughout the study, all experiments has been run in duplicate, unless otherwise mentioned. Raw results were presented as mean ± SD. The data obtained were analyzed using the unpaired *t* test data. *P* values of <0.05 were considered to be statistically significant.

Evaluation of intrinsic disorder propensity

The per-residue intrinsic disorder propensities of human and avian defensins analyzed in this study (see Table 5) were evaluated by the PONDR® FIT metapredictor, which is one of the most accurate disorder predictors.[52] Charge-hydropathy plot, represents an approach for classification of an entire protein as mostly ordered or disordered.[53, 54] Here, a protein is presented as a point within the charge-hydropathy phase space with the coordinates of this point being parameters calculated from the amino acid sequence, absolute mean net charge <R> and mean hydropathy <H>. This CH-plot represents the charge-hydrophacy phase space, where ordered and disordered proteins occupy two different areas and can be separated by a boundary line, <R> = 2.785<H> - 1.151, with ordered and intrinsically disordered proteins being located below and above this boundary, respectively.[54]

Author Contributions

EHM and HAA collected and analyzed data, contributed to discussion, and participated in writing of the manuscript. VNU conducted computational analysis, contributed to discussion, and wrote, reviewed and edited the manuscript. EMR conceived the idea, supervised the project, organized and analyzed data, contributed to discussion, and wrote the manuscript.

Competing Financial Interests

The authors declare no competing financial interests.

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765 ## Figure Legends

766 **Figure 1.** Schematic of synchronized infectivity assays of different defensins
767 scenarios.

768 **Figure 2. A**. Huh7.5 cell line **(left side**) and PBMCs (**right side**) protection from
769 HCV infection by defensins. The cells were incubated with the defensins and then
770 exposed to HCV particles. **B**. Neutralization potentials of defensins against HCV
771 particles on HuH 7.5 cell line (**left side**) and PBMCs (**right side**). Different types and
772 concentrations defensins were incubated with HCV particles then used for HuH7.5 or
773 PBMCs infection. **C**. Effect of intracellular treatment with defensins on HCV
774 replication in Huh7.5 cell line (**left side**) and PBMCs (**right side**). HCV infected
775 Huh7.5 cell line or PBMCs were treated with different type and concentrations of
776 defensins. In all plots, lane 1 pointed the DNA leader, lanes 2 and 3 show negative
777 (non-infected Huh7.5 or PBMC) and positive (infected Huh7.5 or PBMC) control
778 samples, respectively, lanes 4-6 show the effects of human natural α–defensins (**a**) and
779 human recombinant β-defensin (**b**) at concentrations 50, 20, 10 µg/ml. Avian β-
780 defensins AvBD-4 (**c**), AvBD-7 (**d**), AvBD-12 (**e**) concentration 250 µg/ml (lanes 4),
781 20 µg/ml (lane 5), and 10 µg/ml (lane 6). Right arrow heads pointed Rulc internal
782 control (upper) and HCV amplified fragment 174 bp (lower). Lane 7 contain the result
783 of camel lactoferrin (cLac) at 500 µg/ml as positive control.

784 **Figure 3**. Intrinsic disorder status of defensins analyzed in this study. The intrinsic
785 disorder propensities of human α-defensins (HNP-1, HNP-2, HNP-3, and HNP-4) (**A**),
786 β-defensins (RHBD-1, RHBD-2, RHBD-3, RHBD-4, RHBD-5, and RHBD-116) (**B**),
787 and avian β-defensins (AvBD-4, AvBD-7, and AvBD-4) (**C**) were evaluated by one of
788 the more accurate per-residue meta-predictors of disorder, PONDR® FIT. In pot **B**, all

789 sequences of the human β-defensins were aligned to have their first Cys residue at the
790 position 40. The predispositions of these proteins to be ordered or disordered as a
791 whole were evaluated using a binary disorder predictor CH-plot (**D**).

792

793 **Table 1.** Cell viability by MTT method.

	PBMCs viability %		**Huh7.5 cells viability %**	
	50µg/ml	250µg/ml	50µg/ml	250µg/ml
Control	100	100	100	100
α-defensin	99	99	99	99
β-defensin	95	94	94	94
AvBD-4	95	95	94	94
AvBD-7	94	95	94	95
AvBD-12	95	94	95	93

794
795

796 **Table 2.** Detection of HCV RNA in the infected Huh7.5 cells or PBMCs[a] in the
797 experiments on the analysis of the cell protection potential of defensins

Protein	Protein conc. (µg/ml)	Calc. conc. (IU/ml)	Relative activity (%)
Control	Positive	250.000 (250.000)	0.00 ± 0.01 (0.0 ± 0.01)
	Negative	0.0 (0.0)	100 ± 0.0 (100 ± 0.0)
α-Defensins	10	0.0 (0.0)	100 ± 0.0** (100 ± 0.0**)
	20	0.0 (0.0)	100 ± 0.0** (100 ± 0.0**)
	50	0.0 (0.0)	100 ± 0.0** 100 ± 0.0**
β-Defensins	10	101.910 (50.750)	59.24 ± 0.02* (79.70 ± 0.01*)
	20	0.0 (0.0)	100 ± 0.0** (100 ± 0.0**)
	50	0.0 (0.0)	100 ± 0.0** (100 ± 0.0**)
Avian defensin AvBD-4	10	221.345 (110.345)	11.46 ± 0.02 (55.86 ± 0.02*)
	20	226.987 (98.765)	9.205 ± 0.009 (60.49 ± 0.01*)
	250	0.0 (0.0)	100 ± 0.0** (100 ± 0.0**)
Avian defensin AvBD-7	10	120.628 (98.443)	51.75 ± 0.01* (60.627 ± 0.005*)
	20	97.539 (81.199)	60.98 ± 0.01* (67.520 ± 0.004*)
	250	0.0 (0.0)	100 ± 0.0** (100 ± 0.0**)
Avian defensin AvBD-12	10	89.850 (43.561)	64.06 ± 0.02* (82.58 ± 0.01*)
	20	55.867 (33.917)	77.653 ± 0.005* (86.43 ± 0.01*)
	250	0.0 (0.0)	100 ± 0.0** (100 ± 0.0**)
cLac	500	0.0 (0.0)	100 ± 0.0** (100 ± 0.0**)

798

799 [a] Results of the real-time-PCR analysis of the cellular protection activity of human α-
800 and β-defensins and avian defensins against HCV entry in comparison with that of the
801 camel lactoferrin at concentration of 500 µg/ml. Uninfected Huh7.5 (or PBMCs) cells
802 and infected Huh7.5 cells (or PBMCs) with HCV were used as negative and positive
803 controls, respectively. Single (*) or double asterisk (**) indicates significant or highly
804 significant differences compared to the positive control. Here, $P<0.05$ was considered
805 as statistically significant.

806

807 **Table 3.** Detection of HCV RNA in the infected Huh7.5 cells or PBMCs[a] in the
808 experiments on the analysis of the neutralization potential of defensins

Protein	Protein conc. (μg/ml)	Calc. conc. (IU/ml)	Relative activity (%)
Control	Positive	250.000 (250.000)	0.00 ± 0.01 (0.0 ± 0.01)
	Negative	0.0 (0.0)	100 ± 0.0 (100 ± 0.0)
α-Defensins	10	0.0 (0.0)	100 ± 0.0** (100 ± 0.0**)
	20	0.0 (0.0)	100 ± 0.0** (100 ± 0.0**)
	50	0.0 (0.0)	100 ± 0.0** (100 ± 0.0**)
ß-Defensins	10	0.0 (0.0)	100 ± 0.0** (100 ± 0.0**)
	20	0.0 (0.0)	100 ± 0.0** (100 ± 0.0**)
	50	0.0 (0.0)	100 ± 0.0** (100 ± 0.0**)
Avian Defensin AvBD-4	10	201.561 (210.440)	17.376 ± 0.008 (15.82 ± 0.01)
	20	196.478 (196.967)	21.409 ± 0.003 (21.21 ± 0.02)
	250	0.0 (0.0)	100 ± 0.0** (100 ± 0.0**)
Avian Defensin AvBD-7	10	211.231 (215.883)	15.51 ± 0.02 (13.647 ± 0.005)
	20	167.698 (157.765)	32.921 ± 0.007 (36.894 ± 0.009)
	250	0.0 (0.0)	100 ± 0.0** (100 ± 0.0**)
Avian Defensin AvBD-12	10	187.911 (181.561)	24.8356 ± 0.006 (27.3756 ± 0.004)
	20	157.778 (150.671)	36.8888 ±0.006 (39.7316 ± 0.014)
	250	0.0 (0.0)	100 ± 0.0** (100 ± 0.0**)
cLac	500	0.0 (0.0)	100 ± 0.0** (100 ± 0.0**)

809

810 [a] Results of the real-time-PCR analysis of the neutralizing activity of human α- and β-
811 defensins and avian defensins against HCV in comparison with that of camel
812 lactoferrin at concentration of 500 μg/ml. Uninfected Huh7.5 cells (or PBMCs) and
813 infected Huh7.5 cells (or PBMCs) with HCV were used as negative and positive,
814 respectively.

815

816 **Table 4.** Detection of the HCV RNA in infected Huh7.5 cells or infected PBMCs[a] in
817 the experiments on the intracellular treatment potential of defensins

Protein	Protein conc. (µg/ml)	Calc. conc. (IU/ml)	Relative activity (%)
Control	Positive	250.000 (250.000)	0.00 ± 0.01 (0.0 ± 0.01)
	Negative	0.0 (0.0)	100 ± 0.0 (100 ± 0.0)
α-Defensins	10	0.0 (0.0)	100 ± 0.0** (100 ± 0.0**)
	20	0.0 (0.0)	100 ± 0.0** (100 ± 0.0**)
	50	0.0 (0.0)	100 ± 0.0** (100 ± 0.0**)
ß-Defensins	10	0.0 (0.0)	100 ± 0.0** (100 ± 0.0**)
	20	0.0 (0.0)	100 ± 0.0** (100 ± 0.0**)
	50	0.0 (0.0)	100 ± 0.0** (100 ± 0.0**)
Avian Defensin AvBD-4	10	231.221 (210.561)	7.511 ± 0.005 (15.776 ± 0.007)
	20	224.326 (201.891)	10.27 ± 0.01 (19.24 ± 0.02)
	250	201.749 (193.279)	19.30 ± 0.02 (22.681 ± 0.005)
Avian Defensin AvBD-7	10	199.870 (209.435)	20.05 ± 0.02 (16.23 ± 0.01)
	20	215.682 (196.543)	13.72 ± 0.02 (21.38 ± 0.01)
	250	197.988 (188.698)	20.805 ± 0.009 (24.521 ± 0.008)
Avian Defensin AvBD-12	10	212.593 (200.675)	14.96 ± 0.02 (19.73 ± 0.01)
	20	201.994 (184.767)	19.20 ± 0.02 (26.093 ± 0.005)
	250	197.381 (173.491)	21.048 ± 0.008 (30.604 ± 0.009)
cLac	500	0.0	100 ± 0.0** (100 ± 0.0**)

818

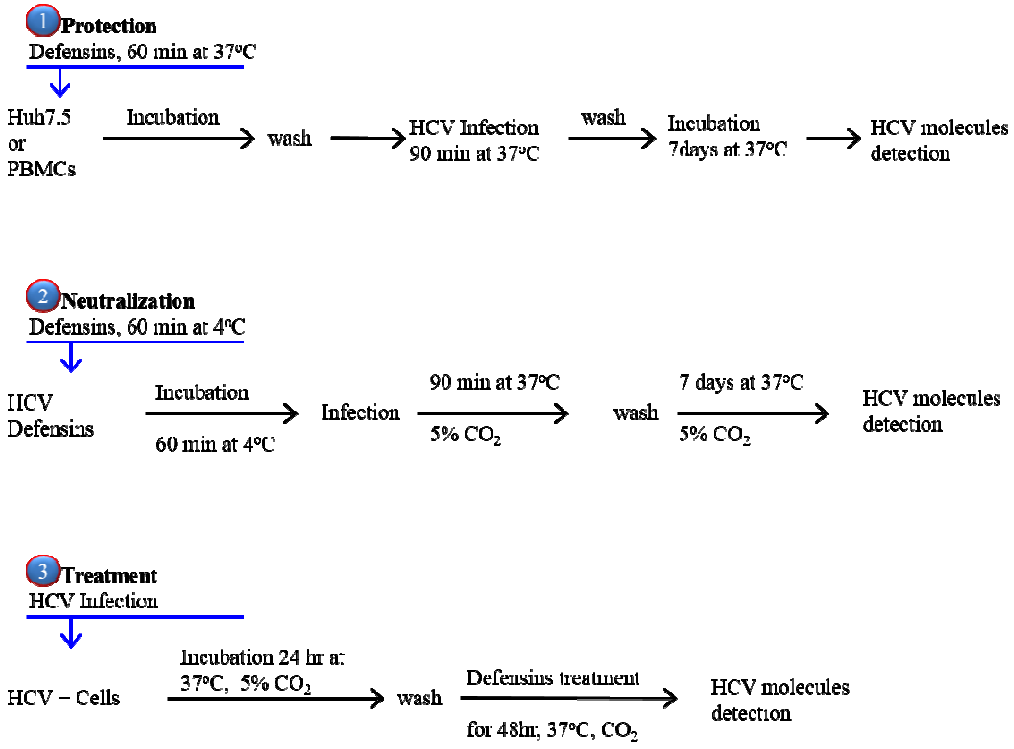
819 [a] Real-time-PCR results of human alpha and beta-defensins, and avian defensins
820 intracellular treatment activity against HCV in comparison with camel lactoferrin at
821 concentration of 500µg/ml. Uninfected Huh7.5 cells and infected Huh7.5 cells with
822 HCV were used as negative and positive, respectively.

823

824 **Table 5**. Basic physico-chemical properties and amino acid sequences of defensins
825 used in this study

Name	pI	Molecular weight	Mean disorder score	Sequence
Human α-defensins (human neutrophil polypeptides, HNPs)				
HNP-1	8.68	3448.09	0.45±0.26	ACYCRIPACI AGERRYGTCI YQGRLWAFCC
HNP-2	8.67	3377.01	0.47±0.26	CYCRIPACIA GERRYGTCIY QGRLWAFCC
HNP-3	8.67	3377.01	0.47±0.26	CYCRIPACIA GERRYGTCIY QGRLWAFCC
HNP-4	8.70	3830.55	0.40±0.28	VCSCRLVFCR RTELRVGNCL IGGVSFTYCC TRVD
Recombinant human β-defensins (RHBDs)				
RHBD-1	8.87	3934.57	0.40±0.28	DHYNCVSSGG QCLYSACPIF TKIQGTCYRG KAKCCK
RHBD-2	9.30	4334.24	0.38±0.26	GIGDPVTCLK SGAICHPVFC PRRYKQIGTC GLPGTKCCKK P
RHBD-3	10.08	5161.20	0.52±0.27	GIINTLQKYY CRVRGGRCAV LSCLPKEEQI GKCSTRGRKC CRRKK
RHBD-4	9.27	5988.91	0.60±0.30	EFELDRICGY GTARCRKKCR SQEYRIGRCP NTYACCLRKW DESLLNRTKP
RHBD-5	8.26	5783.67	0.63±0.20	GLDFSQPFPS GEFAVCESCK LGRGKCRKEC LENEKPDGNC RLNFLCCRQR I
RHBD-116	8.58	11509.77	0.60±0.36	MGSSHHHHHH SSGLVPRGSH MGSGLFRSHN GKSREPWNPC ELYQGMCRNA CREYEIQYLT CPNDQKCCLK LSVKITSSKN VKEDYDSNSN LSVTNSSSYS HI
RHBD-116 without His-Tag	8.71	11544.33	0.44±0.34	MSVMKPCLMT IAILMILAQK TPGGLFRSHN GKSREPWNPC ELYQGMCRNA CREYEIQYLT CPNDQKCCLK LSVKITSSKN VKEDYDSNSN LSVTNSSSYS HI
Avian synthetic β-defensins				
AvBD-4	8.24	7130.49	0.26±0.22	MKILCLLFAV LLFLFQAAPG SADPLFPDTV ACRTQGNFCR AGACPPTFTI SGQCHGGLLN CCAKIPAQ
AvBD-7	8.65	7278.51	0.33±0.21	MRILFFLVAV LFFLFQAAPA YSQEDADTLA CRQSHGSCSF VACRAPSVDI GTCRGGKLKC CKWAPSS
AvBD-12	9.50	7162.61	0.25±0.18	MKILCFFIVL LFVAVHGAVG FSRSPRYHMQ CGYRGTFCTP GKCPHGNAYL GLCRPKYSCC RWL

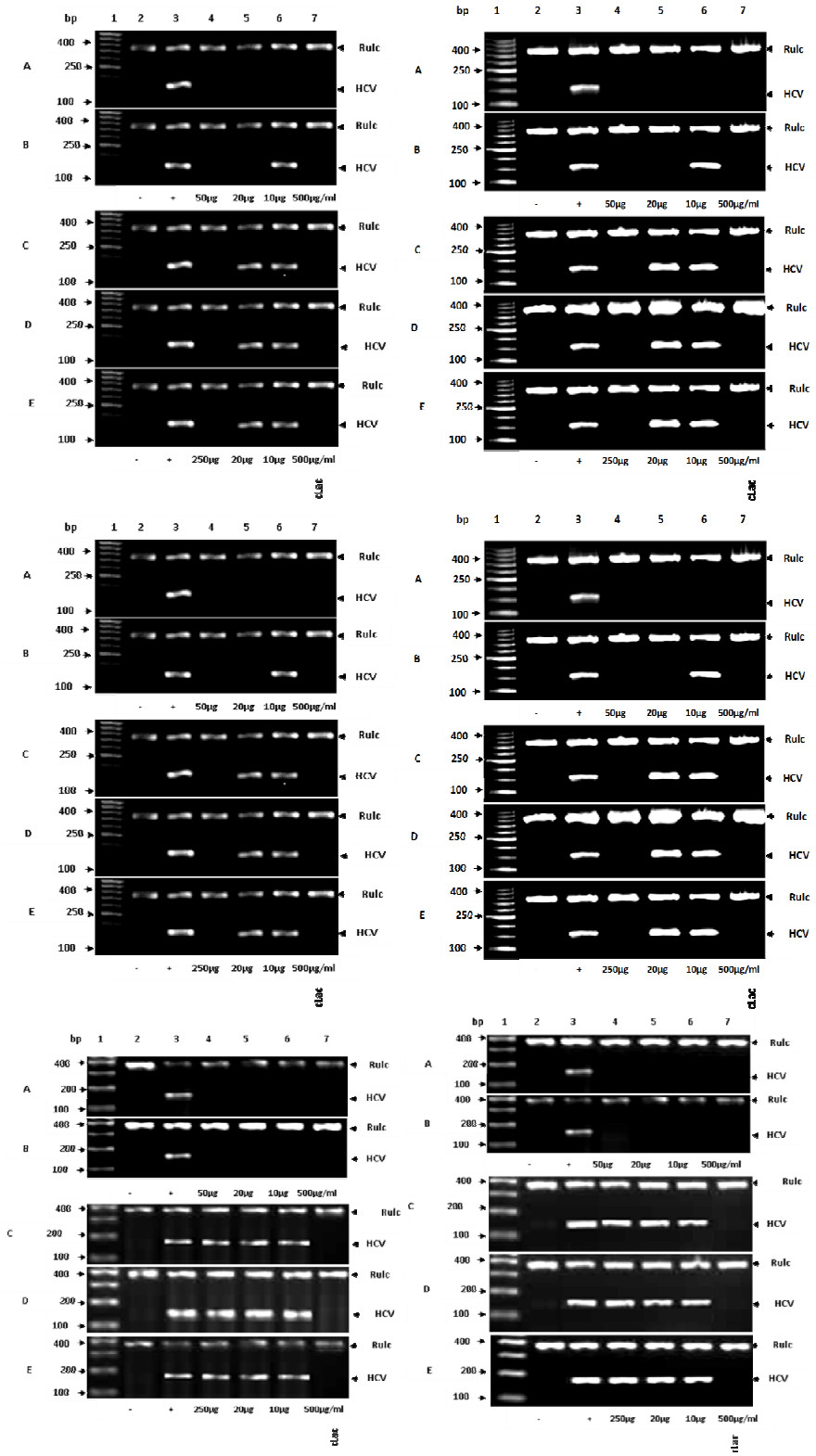
826



827

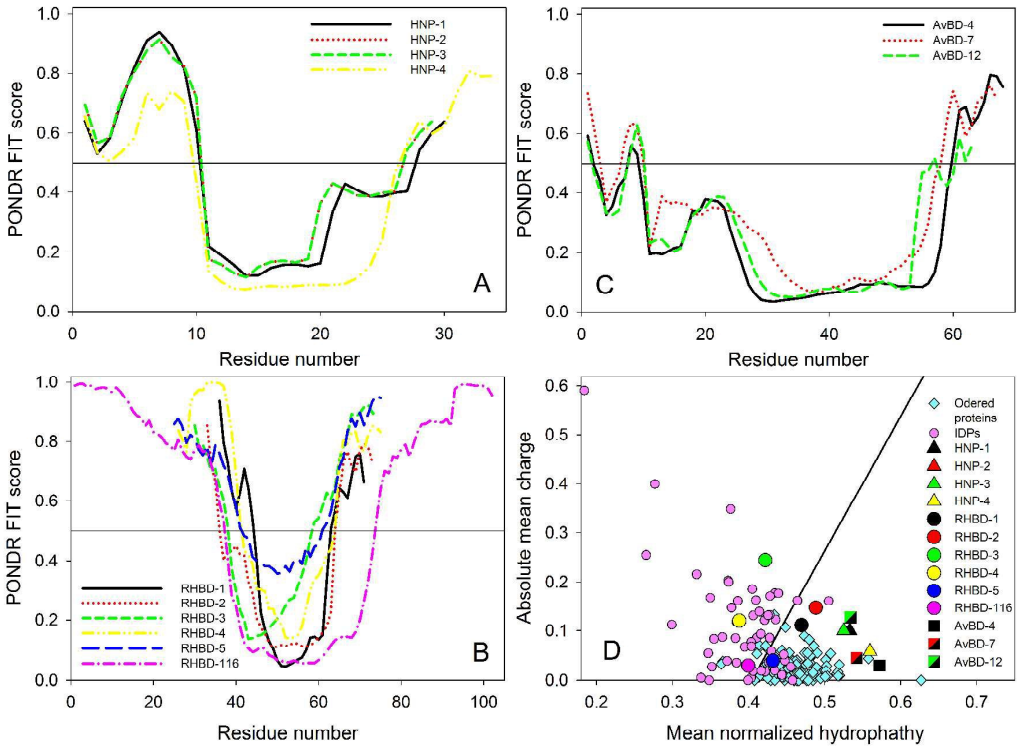
828 Figure 1

829



830

831 Figure 2



832

833 Figure 3